

Increased type 1 plasminogen activator inhibitor gene expression in atherosclerotic human arteries

(atherosclerosis/fibrinolysis/*in situ* hybridization)

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ABSTRACT Decreased fibrinolytic capacity has been suggested to accelerate the process of arterial atherogenesis by facilitating thrombosis and fibrin deposition within developing atherosclerotic lesions. Type 1 plasminogen activator inhibitor (PAI-1) is the primary inhibitor of tissue-type plasminogen activator and has been found to be increased in a number of clinical conditions generally defined as prothrombotic. To investigate the potential role of this inhibitor in atherosclerosis, we examined the expression of PAI-1 mRNA in segments of 11 severely diseased and 5 relatively normal human arteries obtained from 16 different patients undergoing reconstructive surgery for aortic occlusive or aneurysmal disease. Densitometric scanning of RNA (Northern) blot autoradiograms revealed significantly increased levels of PAI-1 mRNA in severely atherosclerotic vessels (mean densitometric value, 1.7 ± 0.28 SEM) compared with normal or mildly affected arteries (mean densitometric value, 0.63 ± 0.09 SEM; $P < 0.05$). In most instances, the level of PAI-1 mRNA was correlated with the degree of atherosclerosis. Analysis of adjacent tissue sections from the same patients by *in situ* hybridization demonstrated an abundance of PAI-1 mRNA-positive cells within the thickened intima of atherosclerotic arteries, mainly around the base of the plaque. PAI-1 mRNA could also be detected in cells scattered within the necrotic material and in endothelial cells of adventitial vessels. In contrast to these results, PAI-1 mRNA was visualized primarily within luminal endothelial cells of normal-appearing aortic tissue. Our data provide initial evidence for the increased expression of PAI-1 mRNA in severely atherosclerotic human arteries and suggest a role for PAI-1 in the progression of human atherosclerotic disease.

The fibrinolytic potential of blood is regulated by the action of specific plasminogen activator inhibitors (PAIs). Endothelial or type 1 PAI (PAI-1) is a fast-acting inhibitor of both urokinase- and tissue-type plasminogen activators (1) and appears to have a major clinical relevance. Deficiencies in its activity result in an increased likelihood of hemorrhagic events (2), whereas increased levels are frequently observed in association with thrombotic phenomena. For example, elevations in plasma PAI-1 activity have been demonstrated in patients with deep venous thrombosis (3, 4) and disseminated intravascular coagulation (5–7). In addition, increased PAI-1 levels have been observed in patients with coronary artery disease (8, 9) and angina pectoris (10) and in young (11) and old (12) survivors of myocardial infarction, suggesting that PAI-1 may represent a risk factor for acute coronary thrombosis (reviewed in ref. 13).

Positive correlations have also been established between plasma PAI-1 levels and known risk factors for the develop-

ment of atherosclerosis. These include obesity (14), noninsulin-dependent diabetes (15), hyperinsulinemia (16), and hypertriglyceridemia (11, 16). Recently, PAI-1 was found to be predictive of coronary atherosclerosis in patients with glucose intolerance and presenting with previous myocardial infarction (17). However, in patients with no prior history of diabetes or hyperlipidemia, plasma PAI-1 activity was not found to correlate with the severity of coronary atherosclerosis and did not differ significantly between patients and healthy controls (18). Collectively, these data suggest that systemic alterations in PAI-1 activity may contribute to the development of atherosclerosis but do not constitute an independent risk factor in this regard.

Since the localized synthesis and release of PAI-1 by cells within vascular tissues could also contribute to this process, we undertook an analysis of PAI-1 gene expression in sections of atherosclerotic human arteries. In this report we provide initial evidence that the expression of PAI-1 mRNA is increased in atherosclerotic versus normal-appearing arterial tissues. Further, we show that its expression in atherosclerotic tissues is localized predominantly to mesenchymal-appearing neointimal cells, suggesting that PAI-1 gene expression may be linked to the cellular proliferative response characteristic of developing atherosclerotic lesions.

METHODS

Tissue Preparation and Evaluation. Specimens for analysis were obtained with informed consent from 16 patients undergoing vascular reconstructive surgery for aortic occlusive or aneurysmal disease. The five normal-appearing segments obtained for these studies (see below) originated from the donor site of the aorta prior to constructing the proximal anastomosis of an aortoiliac or aortofemoral bypass. Segments of arterial wall collected during surgery were washed to remove blood clots and immediately evaluated by macroscopic criteria for the degree of atherosclerotic involvement. Arterial segments showing no evidence of atherosclerosis or exhibiting only a mild involvement of the wall (i.e., mild thickening or fatty streaks) were classified as normal-appearing, while segments characterized by a thickened wall with ulcerations and calcifications were classified as severely atherosclerotic. Of the severely atherosclerotic samples, 9 of 11 were segments of aneurysm wall (8 abdominal aneurysms, 1 femoral aneurysm). The other 2 were removed from non-dilated aortas. Prior to storage, the tissue samples were divided into parallel sections for Northern blot analysis, *in*

Abbreviations: PAI, plasminogen activator inhibitor; PAI-1, type 1 PAI; GAPDH, glyceraldehyde-6-phosphate dehydrogenase; PDGF, platelet-derived growth factor; TF, tissue factor.

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situ hybridization, and histopathologic examination by light microscopy. To confirm the macroscopic classification of atherosclerotic involvement assigned to each specimen, a representative section of each arterial segment was examined by routine light microscopy, or polarized light microscopy when necessary, for the identification of collagen. The macroscopic and microscopic findings were in complete concordance in all cases.

Northern Blot Analysis. Tissue segments for Northern blot analysis were stored in liquid nitrogen. For the isolation of total RNA, the tissues were pulverized on dry ice, and total RNA was extracted by the acid guanidinium thiocyanate-phenol/chloroform method (19). The concentration of RNA was determined by sample absorbance at 260 nm. For Northern blotting, total RNA samples (30 μ g) were electrophoresed under denaturing conditions and transferred to nylon membrane (Biotrans; ICN) as described (20). Equal loading and transfer of the RNA was verified by visualization of the ethidium bromide-stained RNA in the membrane after transfer. The blots were prehybridized for 30 min in 50 mM Pipes buffer, pH 6.8/100 mM NaCl/20 mM Na_2HPO_4 , 30 mM NaH_2PO_4 /1 mM EDTA/5% SDS and then hybridized in this solution supplemented with 10^6 cpm of radiolabeled probe (see below) per ml for 16 hr at 65°C. The blots were then washed four times for 15 min each with prewarmed (65°C) $0.67\times$ SSC ($1\times$ SSC is 150 mM NaCl/15 mM sodium citrate) containing 5% SDS and autoradiographed at -70°C on Kodak XAR-5 film with intensifying screens.

In Situ Hybridization. Tissue segments for *in situ* hybridization were fixed in paraformaldehyde (4% wt/vol in phosphate-buffered saline) at 4°C overnight, incubated for 4 hr in 15% sucrose at 4°C, and frozen in OCT compound (Tissue Tek, Miles). Tissue sections (4–10 μ m) were prepared and pretreated sequentially with paraformaldehyde (10 min at 4°C) and proteinase K (1 μ g/ml in 500 mM NaCl/10 mM Tris-HCl, pH 8.0, for 10 min at 23°C). The slides were prehybridized for 1 hr at 42°C in 100 μ l of prehybridization buffer [50% (wt/vol) formamide/0.3 M NaCl/20 mM Tris-HCl, pH 8.0/5 mM EDTA/0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin/10% (wt/vol) dextran sulfate/10 mM dithiothreitol]. Prehybridization buffer (20 μ l) containing 0.6×10^6 cpm of radiolabeled probe (see below) and yeast tRNA at 2.5 mg/ml was then added, and the slides were hybridized for 16 hr at 55°C. After hybridization, the sections were washed twice with $2\times$ SSC (10 min per wash at 23°C), treated with RNase A (10 μ g/ml in 500 mM NaCl/10 mM Tris-HCl, pH 7.6, for 30 min at 23°C), and washed twice with $2\times$ SSC (10 min per wash at 23°C) and once with $0.1\times$ SSC at 60°C for 2 hr. The sections were then washed twice in $0.5\times$ SSC without 2-mercaptoethanol (10 min per wash at 23°C) and dehydrated in a graded alcohol series containing 0.3 M $\text{CH}_3\text{COONH}_4$. For detection of the hybridization signal, the sections were coated with NTB2 emulsion (Kodak) diluted 1:2 in distilled water and exposed in the dark at 4°C for 4 weeks. The slides were then developed for 2 min in D19 developer (Kodak), fixed, washed three times in distilled water (5 min per wash), and counterstained with hematoxylin and eosin by standard procedures.

Probes. For Northern analysis, a DNA fragment corresponding to bases 1–1249 of the human PAI-1 cDNA (21) or a plasmid containing a cDNA insert for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; American Type Culture Collection) were radiolabeled to a specific activity of $>5 \times 10^8$ cpm/ μ g by the random primer technique (22) in the presence of [α - ^{32}P]dGTP (>3000 Ci/mmol, Amersham; 1 Ci = 37 GBq). Rehybridization with the GAPDH probe was performed without prior stripping of the blots. For *in situ* hybridization, a 1.3-kilobase (kb) human PAI-1 cDNA fragment, subcloned in the vector pGEM-3 (Promega), was used as a template for *in vitro* transcription of antisense or sense

probes by SP6 or T7 RNA polymerase, respectively (Promega) in the presence of uridine 5'-[α -(^{35}S)thio]triphosphate (>1000 Ci/mmol; Amersham).

Quantitation of PAI-1 mRNA Levels. The PAI-1 and GAPDH mRNA autoradiographic signals were quantitated by scanning densitometry with an LKB Ultrosan XL laser densitometer. All densitometric measurements, expressed as absorbance (*A*) units/ mm^2 of peak area, were within the linear range of measurement as established by coanalysis of results with an AMBIS radioanalytic imaging device (Automated Microbiology Systems, San Diego) as described (23). Densitometry units for PAI-1 mRNA (see Fig. 2) were obtained by dividing the total *A* units/ mm^2 obtained for both PAI-1 mRNA species (i.e., the sum of the areas under the respective peaks) by the corresponding *A* units/ mm^2 obtained for GAPDH mRNA. Statistical significance of the results was evaluated by the Mann-Whitney rank sum test (24).

RESULTS

Northern Blot Analysis. Northern blot analysis of total RNA prepared from arterial specimens revealed increased levels of PAI-1 mRNA in severely atherosclerotic samples of human arteries in comparison with segments of normal or mildly affected vessels (Fig. 1). Similar results were obtained from Northern analysis of additional samples (3 normal-appearing and 6 severely atherosclerotic vessels; autoradiograms not shown). Both species of PAI-1 mRNA (3.2 and 2.3 kb) were elevated in the severely diseased vessels. Rehybridization of the blot with a GAPDH probe was performed to verify the presence of equal amounts of total RNA in each lane (Fig. 1). The PAI-1 and GAPDH mRNA autoradiographic signals were quantitated by scanning densitometry, and the densitometric value for PAI-1 mRNA was normalized to that of GAPDH as detailed in *Methods*. The normalized data for the 16 samples analyzed is shown in Fig. 2. Statistical analysis revealed that the level of PAI-1 mRNA was significantly increased in vessels with severe atherosclerotic involvement (mean densitometric value, 1.7 ± 0.28 SEM) relative to normal or mildly involved vessels (0.63 ± 0.09 SEM; $P < 0.05$).

In Situ Hybridization. To localize PAI-1 mRNA within the arterial specimens, *in situ* hybridization was performed on parallel tissue sections. In the normal or mildly involved arteries, a strong positive hybridization signal was observed in the majority of luminal endothelial cells, while a somewhat weaker signal was observed in scattered stromal cells of the intima (Fig. 3A *Upper*). No positive signal could be visualized in the media (Fig. 3A *Lower*). In severely atherosclerotic vessels, a strong hybridization signal for PAI-1 mRNA was

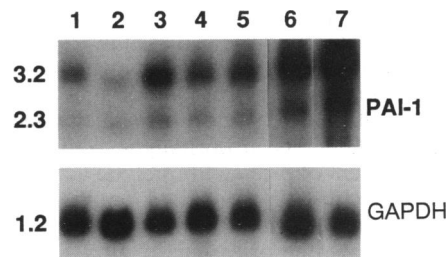


FIG. 1. Northern blot analysis of PAI-1 mRNA in arterial tissues. Total RNA was extracted from normal-appearing or severely atherosclerotic specimens of abdominal aortic wall and analyzed for PAI-1 mRNA by Northern blotting as described in text. Lanes: 1 and 2, normal aortas; 3–7, severely atherosclerotic aortic wall. (*Upper*) The two human PAI-1 transcripts (3.2 kb and 2.3 kb) are indicated. (*Lower*) Signal obtained from rehybridization of the blot with a GAPDH probe, which detects a single mRNA species of 1.2 kb.

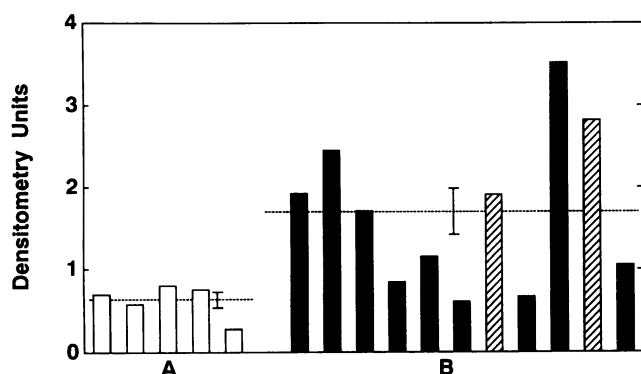


FIG. 2. Quantitative analysis of PAI-1 mRNA expression in arterial tissues. PAI-1 and GAPDH mRNA levels in normal-appearing or severely atherosclerotic arterial specimens were determined by Northern blot analysis of total RNA as depicted in Fig. 1. The mRNA levels were quantitated by densitometric analysis of the autoradiograms with subsequent normalization of the PAI-1 mRNA values to those of GAPDH as described in text. (A) Normal or mildly affected aortas. (B) Severely atherosclerotic arteries. Black bars represent segments of aneurysm wall (eight abdominal aortas and one femoral artery); striped bars represent samples of nondilated abdominal aorta. Error bars shown indicate SEM. Mean value for A = 0.63 ± 0.09 and for B = 1.7 ± 0.28 .

observed within the thickened intima and in the endothelium of adventitial arterioles but not in the luminal endothelium (Fig. 3B). In general, the luminal endothelium of the severely diseased vessels (Fig. 3B *Top Left*) exhibited less positive signal than that of the normal aortic endothelium (Fig. 3A *Upper*). Within atherosclerotic plaques containing cholesterol clefts, fibrin, and amorphous debris, clusters of cells with a very prominent hybridization signal for PAI-1 mRNA were identified most frequently at the base and sides of the plaque (Fig. 3B *Top and Middle*). A few PAI-1 mRNA-positive cells could be found within the necrotic plaque material (Fig. 3B *Middle Left*). Some atherosclerotic plaques were associated with aggregates of inflammatory cells, and rare cells within these aggregates demonstrated a positive PAI-1 mRNA signal (Fig. 3B *Middle Right*). In the adventitia of severely atherosclerotic aortas, extramural arterioles exhibited a positive hybridization signal in the arteriolar endothelium (Fig. 3B *Bottom*). In addition, scattered extravascular foci containing PAI-1 mRNA could be seen in the fibrous and adipose tissue of the adventitia. Control hybridizations using a sense PAI-1 riboprobe were uniformly negative (data not shown).

DISCUSSION

Intravascular or mural thrombosis is a frequent histologic feature of atherosclerotic lesions and appears to play a role in the intimal thickening and fibrosis characteristic of advanced lesions (25). In the prothrombotic state, intravascular thrombosis and fibrin deposition may be facilitated by either systemic or local deficits in fibrinolytic activity. Although systemic impairment of plasma fibrinolytic activity has been noted in patients with symptomatic coronary occlusive disease (reviewed in ref. 13), the majority of patients with generalized arterial atherosclerosis exhibit normal plasma fibrinolytic profiles (26). However, since localized alterations in fibrinolytic activity could also influence this process, we evaluated PAI-1 gene expression within segments of atherosclerotic human arteries.

Northern blot analysis of total RNA isolated from arterial segments (Fig. 1) revealed a clear trend between the degree of atherosclerosis and PAI-1 gene expression. Both species of PAI-1 mRNA (3.2 and 2.3 kb), which contain identical

codogenic regions but differ in the length of their 3' untranslated regions (21), were increased in 11 severely atherosclerotic vessels compared with 5 normal-appearing or mildly involved vessels. The increase of PAI-1 mRNA appeared to be a function of atherosclerotic and not aneurysmal involvement, since 2 specimens from undilated atherosclerotic vessels demonstrated the same characteristic pattern of PAI-1 mRNA expression. Quantitative analysis of data obtained from the 16 arterial specimens (Fig. 2) indicated that the mean PAI-1 mRNA level was significantly increased in severely atherosclerotic arteries (mean densitometric value, 1.7 ± 0.28 SEM) relative to normal-appearing vessels (mean densitometric value, 0.63 ± 0.09 SEM; $P < 0.05$).

In situ hybridization analysis of the severely diseased vessels (Fig. 3B) revealed an intense hybridization signal for PAI-1 mRNA in cells clustered about the base and sides of the plaque. The appearance and distribution of these cells resembles that of the mesenchymal-appearing intimal cells (MICs), assumed to be modified smooth muscle cells (27), which predominate in advanced atherosclerotic lesions. The nature of the stimulus for the increased expression of PAI-1 mRNA by these cells is unclear. Platelet-derived growth factor (PDGF) is the main chemotactic and mitogenic factor for smooth muscle cells and appears to be significantly involved in atherogenesis (28). Previous *in situ* hybridization studies have demonstrated mRNA for both the PDGF-A and -B chains and the PDGF-b receptor (29) in MICs within atherosclerotic plaques. In human coronary atheromas, tissue factor (TF) mRNA and protein were visualized in macrophages and monocytes adjacent to the cholesterol clefts and within cells resembling MICs (30). In preliminary experiments, PAI-1 mRNA was colocalized to cells expressing PDGF and PDGF receptor (31). *In vitro*, PDGF was shown to induce TF mRNA in murine 3T3 cells (32) and to stimulate PAI-1 gene expression and biosynthesis in cultured bovine vascular smooth muscle cells (33). Collectively, these observations suggest that PDGF may act in an autocrine or paracrine fashion to increase PAI-1 and TF expression in atheromatous vessels.

In situ hybridization of normal-appearing or mildly involved aortic segments (Fig. 3A *Upper*) revealed a distinct positive hybridization signal for PAI-1 mRNA in luminal aortic endothelial cells, while no hybridization signal could be detected in the endothelium of adventitial vessels within these segments (Fig. 3A *Lower Right*). A similar pattern of PAI-1 gene expression was observed in normal-appearing tissues obtained from the same patients as the severely atherosclerotic vessels (data not shown). The presence of PAI-1 mRNA in luminal aortic endothelial cells and its absence in endothelial cells of adventitial vessels may simply reflect phenotypic differences between large and small vessel endothelium. However, other observations raise the possibility that endothelial PAI-1 mRNA expression *in vivo* may be characteristic of an activated endothelium. For example, in human colon adenocarcinomas, PAI-1 mRNA was shown to be present in endothelial cells of vessels immediately surrounding invasive tumor glands, while no hybridization signal could be visualized in biopsies of normal human colon (34). Similarly, in murine renal tissues, a strong hybridization signal for PAI-1 mRNA was evident in endothelial cells after treatment of mice with lipopolysaccharide, while no signal could be detected in the endothelium of normal mice (35). *In vitro* the expression of PAI-1 mRNA in cultured aortic endothelial cells is dramatically increased in response to inflammatory mediators, including interleukin 1 and tumor necrosis factor α (20, 36) as well as transforming growth factor β (20), which is released from activated platelets during thrombosis (37). The expression of PAI-1 mRNA in luminal endothelial cells of normal-appearing aortic tissue (Fig. 3A) may thus reflect localized activation of the endothelium by

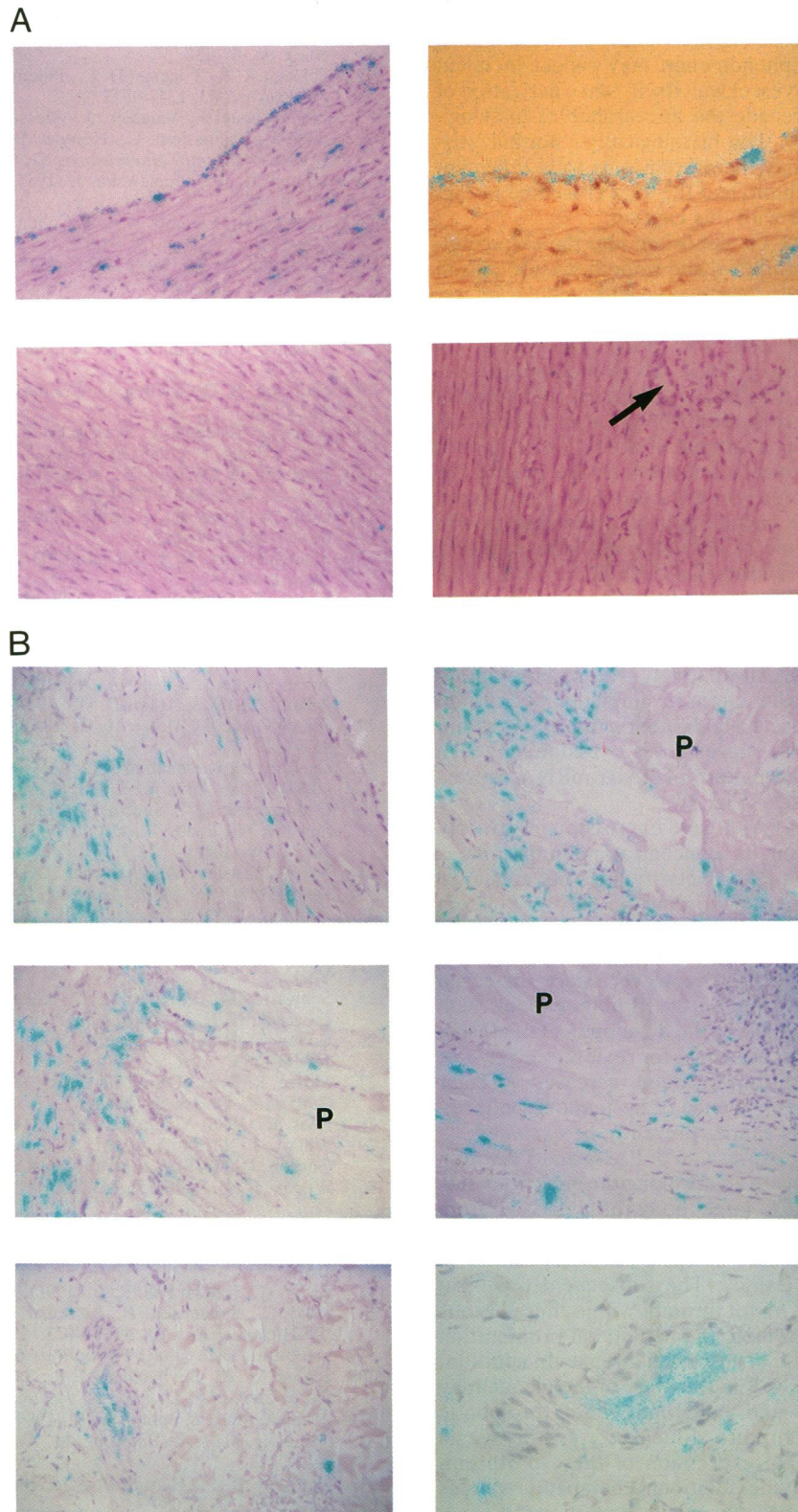


FIG. 3. *In situ* hybridization analysis of PAI-1 mRNA expression in arterial tissues. Panels show photomicrographs of sections of human aortas following *in situ* hybridization with an anti-sense human PAI-1 riboprobe, and counterstaining with hematoxylin and eosin as described in text. Sections shown in A and B were derived from two separate patients. Light blue clustered dots represent intracellular PAI-1 mRNA. (A) Normal-appearing aorta. (Upper Left) Positive signal in luminal endothelial cells with scattered signal in cells within the intima. (Upper Right) Strong signal in endothelial cells. (Lower Left) Media with no demonstrable positive signal. (Lower Right) Media and adjacent adventitia with no positive signal. Note the adventitial blood vessel (arrow) with no evidence of positive signal in endothelial cells. (B) Severely atherosclerotic aorta. (Top Left) Note lack of positive signal in most surface (endothelial) cells, the presence of subendothelial fibrocollagenous bands, and aggregates of cells with strong positive signal in the thickened intima. (Top Right and Middle) Three examples of atherosclerotic plaque (P) with underlying highly positive cells. (Bottom Left) Adventitia with scattered positive cells and strong positive signal in arteriolar endothelial cells. (Bottom Right) Higher magnification of the adventitial arteriole shown in Bottom Left. ($\times 105$ except A Upper Right and B Bottom Right, which were $\times 210$.)

humoral mediators released as a consequence of inflammatory or thrombotic processes in the vicinity of the sampling site. Alternatively, this phenomenon may reflect incipient atherogenesis within the vessel wall itself, since activation of the endothelium may precede the appearance of histologically defined lesions (38). The histologically "normal" tissues used in this study may therefore not be truly representative of normal endothelium, and the data presented in Fig. 3A should be interpreted with this caution.

Newly synthesized PAI-1 is deposited into the extracellular matrix of vascular endothelial and smooth muscle cells in culture (39, 40) and appears to preserve extracellular matrix structures from degradation initiated by cellular proteases released during cell migration and tissue remodeling. By limiting extracellular proteolysis in developing atherosclerotic lesions, PAI-1 may thus play a significant role not only in the organization and incorporation of mural thrombi within the plaque but also in the neointimal proliferation of smooth muscle cells and in neovascularization of the plaque. In addition to PDGF, numerous other factors have been implicated in these processes, including basic fibroblast growth factor, which is angiogenic *in vivo* (41) and induces PAI-1 biosynthesis in capillary endothelial cells *in vitro* (42). The presence of basic fibroblast growth factor within the plaque may elicit the endothelial expression of PAI-1 mRNA observed in arterioles and vasa vasorum of the diseased vascular segments (Fig. 3B). Alternatively, the recent demonstration of tumor necrosis factor α immunoreactivity in endothelial, smooth muscle, and monocytic cells within atherosclerotic tissues (43) suggests this cytokine may also contribute to the observed pattern of PAI-1 mRNA expression in the diseased vessels (Fig. 3).

The effect of increased PAI-1 gene expression in severely atherosclerotic vessels on its concentration in plasma is presently unclear. Plasma PAI-1 activity does not appear to correlate with the severity of coronary atherosclerosis in otherwise healthy men (18). Moreover, the majority of patients with generalized arterial atherosclerosis exhibit normal plasma fibrinolytic profiles (26). However, since increased plasma PAI-1 levels have been observed in individuals with metabolic disorders predisposing the development of atherosclerosis (e.g., hypertriglyceridemia; for review, see ref. 13), the existence of a relationship between increased vascular PAI-1 expression and the incidence of systemic hypofibrinolysis in these individuals cannot presently be excluded.

Locally, the coincident expression of PAI-1 and TF within the plaque may both instigate and prolong thrombotic events within the vessel wall. A large body of evidence from histopathologic examination of human atheromas has demonstrated that such events are likely to occur repeatedly in growing atherosclerotic lesions. The subsequent organization and incorporation of mural thrombi within these lesions has been postulated to promote the neointimal proliferation and sclerosis characteristic of advanced disease. In addition, the presence of PAI-1 and TF in the body of the plaque may contribute to occlusive and potentially fatal thrombotic events following plaque rupture. Elucidation of the agents and mechanisms governing expression of these prothrombotic molecules may provide additional therapeutic venues for the control of atherosclerotic and cardiovascular disease.

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